

Macrophages are essential contributors to kidney injury in murine cryoglobulinemic membranoproliferative glomerulonephritis

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Mice transgenic for thymic stromal lymphopoietin (TSLP), under regulation of the lymphocyte-specific promoter *Lck*, develop cryoglobulinemia and membranoproliferative glomerulonephritis (MPGN) similar to the disease in patients. To determine whether infiltrating macrophages, a hallmark of this disease, are deleterious or beneficial in the injury process, we developed *Lck-TSLP* transgenic mice expressing the human diphtheria toxin receptor (DTR) under control of the monocyte/macrophage-restricted *CD11b* promoter (*Lck-TSLP;CD11b-DTR*). Treatment with DT resulted in a marked reduction of monocytes/macrophages in the peritoneal cavity of both *CD11b-DTR* and *Lck-TSLP;CD11b-DTR* mice and marked reduction of macrophage infiltration in glomeruli of *Lck-TSLP;CD11b-DTR* mice. *Lck-TSLP;CD11b-DTR* mice, with or without toxin treatment, had similar levels of cryoglobulinemia and glomerular immunoglobulin deposition as *Lck-TSLP* mice. *Lck-TSLP;CD11b-DTR* mice, treated with toxin, had reduced mesangial matrix expansion, glomerular collagen IV accumulation, expression of the activation marker α -smooth muscle actin and transforming growth factor- β 1 in mesangial cells, and proteinuria compared with control mice. Thus, macrophage ablation confers protection in this model and indicates a predominately deleterious role for macrophages in the progression of kidney injury in cryoglobulinemic MPGN.

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Macrophages are key participants in inflammation, phagocytosis, tissue repair, and remodeling.^{1–4} They are commonly present in glomerulonephritis (GN), and a large influx of monocytes/macrophages is a particular feature of human cryoglobulinemic membranoproliferative GN (MPGN). Although a characteristic feature of this injury, it is still unknown whether the infiltrating monocytes/macrophages fulfill a predominantly beneficial/reparative role in the evolution of this disease or whether they are predominately effectors or promoters of glomerular injury and sclerosis.

We have characterized a mouse model of cryoglobulinemia with consequent development of MPGN, the thymic stromal lymphopoietin (TSLP) transgenic mouse (*Lck-TSLP*), which overexpresses TSLP and has resultant abnormalities in B-cell development.^{5–7} *Lck-TSLP* mice develop mixed cryoglobulinemia and a systemic inflammatory disease that involves kidney, lung, spleen, liver, and skin.^{5–7} These mice develop a renal disease that closely resembles human cryoglobulinemia-associated MPGN. The glomerular injury is characterized by extensive subendothelial capillary and mesangial immune deposits, marked monocyte/macrophage influx, mesangial cell proliferation and matrix expansion, and capillary wall splitting. The renal lesion is evident from about age 30 days in female mice and rapidly progresses to fully developed MPGN by age 50 days. There are also significant lung,^{5–7} liver,^{5–7} and skin lesions.^{8,9} Female mice usually die at 60 days to 70 days, presumably from severe lung disease. Male mice have a similar but more slowly evolving course, in which the disease is fully manifest by 120 days.^{5–7}

A number of approaches to delete monocyte/macrophage populations, including the use of anti-macrophage serum and liposome-encapsulated clodronate, has been used to test the importance of monocytes/macrophages *in vivo* in renal disease processes.^{10–12} Monocyte/macrophage depletion using liposomal clodronate reduced tubulointerstitial inflammation and fibrosis in ischemia/reperfusion injury in the rat and mouse.^{13,14} A unique *CD11b-DTR* transgenic mouse, in which human diphtheria toxin receptor (DTR) is specifically expressed by *CD11b*-expressing cells, allows conditional

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ablation of mouse monocytes/macrophages by administration of DT. These mice have been carefully characterized for specificity of ablation, and used to study peritoneal inflammation, kidney fibrosis, kidney repair and regeneration, lung, liver and pancreas injury, wound healing, and atherosclerosis.^{15–18} In a model of rapidly progressive glomerular injury, injection of DT for 5 days, beginning at days 15–20 after a single administration of nephrotoxic serum in *CD11b-DTR* mice, resulted in markedly reduced glomerular crescents, attenuated tubular injury, and improved renal function.¹⁸ The effect of monocyte/macrophage depletion has not been tested in a model of chronic GN with active and persistent immune complex deposition, such as that occurs in the cryoglobulinemic *Lck-TSLP* mice and in many patients with MPGN. In this study, we demonstrate that macrophages are essential contributors to the progression of GN and that their presence is a deleterious manifestation of injury.

RESULTS

Lck-TSLP; *CD11b-DTR* and *Lck-TSLP* mice develop equivalent spontaneous glomerulonephritis

Lck-TSLP mice and *CD11b-DTR* mice, both on C57BL/6 background, were bred to obtain double-transgenic mice (*Lck-TSLP*; *CD11b-DTR*). Cohorts of C57BL6 wild-type

(WT), *CD11b-DTR*, *Lck-TSLP*, and *Lck-TSLP*; *CD11b-DTR* mice were studied with intraperitoneal administration of 20 ng/g DT body weight or similar volume of phosphate-buffered saline (PBS) from age days 30–50.

CD11b-DTR mice are similar to WT mice with no detectable kidney pathology. The *Lck-TSLP*; *CD11b-DTR* mice have similar levels of serum cryoglobulinemia, measured by cryocrits, as to *Lck-TSLP* mice (data not shown). Both *Lck-TSLP* and *Lck-TSLP*; *CD11b-DTR* mice developed typical features of cryoglobulin-associated MPGN as described previously.⁵ Mice showed progressive kidney injury from age 30 days (*Lck-TSLP*; *CD11b-DTR*-30D) to age 50 days, with extensive mesangial cell proliferation and mesangial matrix expansion as demonstrated by increased glomerular tuft area, glomerular hypercellularity, and increased silver methanamine-stained extracellular matrix (Figures 1 and 2). Mesangial cell activation, assessed by α -smooth muscle actin (α -SMA) expression, was also markedly increased.

Glomerular macrophages in *Lck-TSLP* mice show marked heterogeneity

Day 50 *Lck-TSLP* mice show a fivefold increase in glomerular M ϕ s compared with WT mice: 2.32 ± 0.2 CD68+ cells per glomerular tuft area versus 0.64 ± 0.05 cells in WT mice.

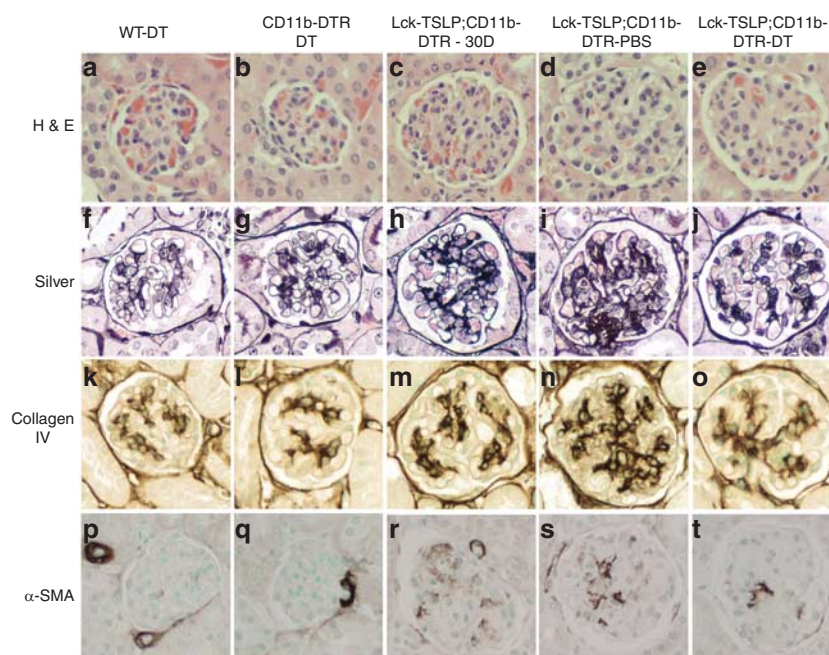


Figure 1 | Macrophage ablation results in amelioration of glomerulonephritis. Histological appearance of glomeruli stained with hematoxylin and eosin (H&E) (a–e), silver methenamine (f–j), and immunohistochemical (IHC) staining for type IV collagen (k–o), and α -smooth muscle actin (α -SMA) (p–t) in wild-type (WT) and *CD11b-DTR* mice of 50 days of age after 20 days diphtheria toxin (DT) treatment (WT-DT and *CD11b-DTR*-DT, respectively), *Lck-TSLP*; *CD11b-DTR* mice at 30 days of age (*Lck-TSLP*; *CD11b-DTR*-30D), and *Lck-TSLP*; *CD11b-DTR* mice at 50 days of age after 20 days of phosphate-buffered saline (PBS) or DT intraperitoneal administration (*Lck-TSLP*; *CD11b-DTR*-PBS and *Lck-TSLP*; *CD11b-DTR*-DT, respectively). *Lck-TSLP*; *CD11b-DTR* mice with PBS treatment show increased glomerular cellularity, mesangial matrix accumulation (black silver staining area increase and type IV collagen expression increase), and mesangial α -SMA expression as compared with WT and *CD11b-DTR* mice, whereas *Lck-TSLP*; *CD11b-DTR* mice with DT treatment demonstrate less hypercellularity, significantly reduced mesangial matrix expansion, and reduced α -SMA expression in glomeruli. Original magnification: $\times 400$.

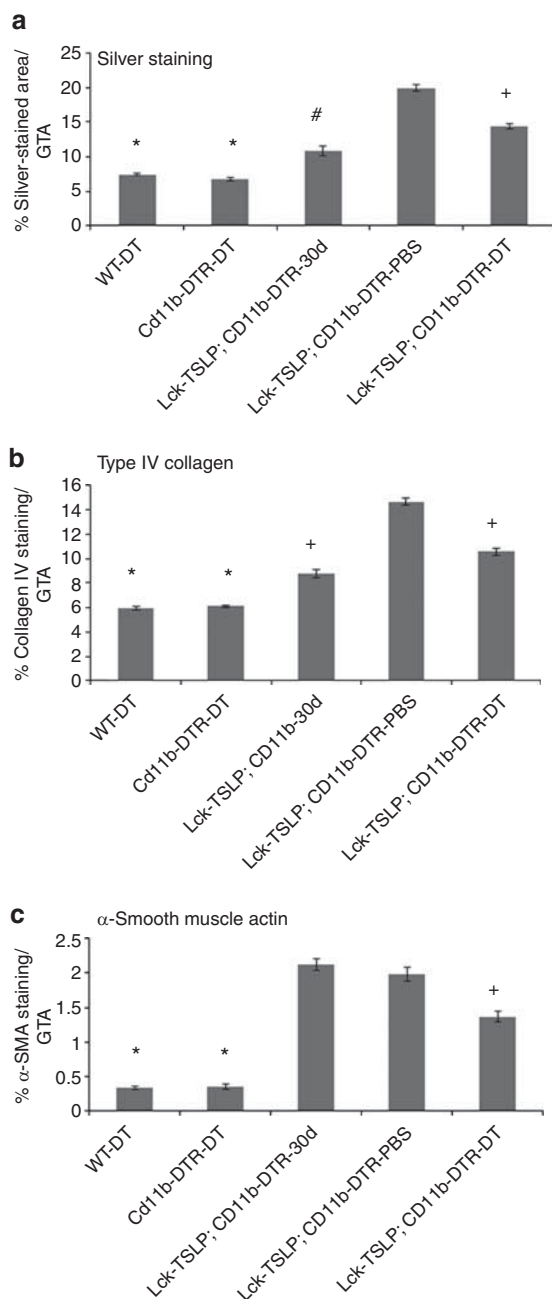


Figure 2 | Macrophage ablation with diphtheria toxin halts progression of disease as shown by morphometric analysis.

(a) Glomerular silver staining matrix; (b) glomerular type IV collagen accumulation; and (c) glomerular α -smooth muscle actin (α -SMA) expression is depicted in wild type (WT), *CD11b-DTR* mice with DT treatment at 50 days of age, *Lck-TSLP; CD11b-DTR* mice at 30 days of age, and *Lck-TSLP; CD11b-DTR* mice at age 50 days, after 20 days of DT or PBS intraperitoneal administration. *Lck-TSLP; CD11b-DTR* mice treated with diphtheria toxin (DT) have significantly reduced expansion of silver-stained extracellular matrix and glomerular type IV collagen expression compared with the phosphate-buffered saline (PBS) control group. A marker of mesangial cell activation, α -SMA is reduced in *Lck-TSLP; CD11b-DTR* mice treated with DT compared to those receiving PBS treatment ($n = 5-6$ in each group). * $P < 0.001$, # $P < 0.01$, + $P < 0.05$ versus *Lck-TSLP; CD11b-DTR-PBS* group. GTA, glomerular tuft area.

In order to gain further insight into the function of these inflammatory macrophages, we characterized them by immunofluorescent staining for recognized markers of M1-type, M2-type, and reparative M ϕ s (Figure 3, Table 1). Strikingly, the CD68+ cells showed high levels of CD11a expression, Ly6C expression, and a proportion expressed CD40, all markers associated with the M1 activated injurious phenotype. Surprisingly, the vast majority of glomerular M ϕ s also expressed some markers associated with M2 or wound healing, namely the scavenger receptor CD206 and galectin-3 (M2). However, other M2-type markers such as integrin β 5 and transferrin receptor were expressed in a minority. Glycoprotein non-metastatic melanoma B-expressing macrophages have been strongly associated with tissue repair in kidney and liver,¹⁹ and a minority of the glomerular M ϕ s also expressed this marker.

Administration of DT reduced blood monocytes and markedly reduced peritoneal and glomerular macrophage infiltration

Flow cytometry analysis of blood monocytes and peritoneal macrophages in *Lck-TSLP; CD11b-DTR* mice showed that blood monocytes expressing CD11b were reduced in the blood in DT-treated (*Lck-TSLP; CD11b-DTR-DT*) ($2.1 \pm 0.6\%$) mice compared with PBS-treated (*Lck-TSLP; CD11b-DTR-PBS*) ($4.3 \pm 0.7\%$) mice (Table 2). Resident peritoneal macrophages were reduced to $4.5 \pm 1.5\%$ of total peritoneal cells in DT-treated mice compared with $22.7 \pm 2.6\%$ in PBS-treated *Lck-TSLP; CD11b-DTR* mice (Table 2). Circulating CD3-expressing T cells were not changed with DT treatment (Table 2).

Both *Lck-TSLP* and *Lck-TSLP; CD11b-DTR* mice have remarkable influx of macrophages in glomeruli as measured by immunohistochemistry detection of Mac-2 (galectin-3) and CD68 epitopes (Figure 3). There are few Mac-2- or CD68-expressing cells in the tubulointerstitium in *Lck-TSLP* and *Lck-TSLP; CD11b-DTR* mice, and these mice do not develop significant tubular atrophy, tubulointerstitial inflammation, or fibrosis.

Lck-TSLP mice treated with either DT or PBS have similar macrophage infiltration in glomeruli. Administration of DT in *Lck-TSLP; CD11b-DTR* mice resulted in a significant reduction in infiltration of macrophages in glomeruli (Figure 4). A significant reduction in glomerular monocyte/macrophage infiltration was also observed when using another marker of macrophages, CD68 (Figure 4g and h). There was an increase in F4/80-positive cells (a marker expressed by monocyte/macrophages preferentially localized to the tubulointerstitium versus glomeruli) infiltrating the tubulointerstitium in *Lck-TSLP; CD11b-DTR* mice compared with WT and *CD11b-DTR* mice (Figure 4i-l). Morphometric analysis demonstrated a trend toward reduction in F4/80-positive interstitial macrophages with DT administration in *Lck-TSLP; CD11b-DTR* ($0.33 \pm 0.13\%$ versus $0.17 \pm 0.09\%$ positive staining per interstitial area), but results were not statistically significant between any of the groups. There was

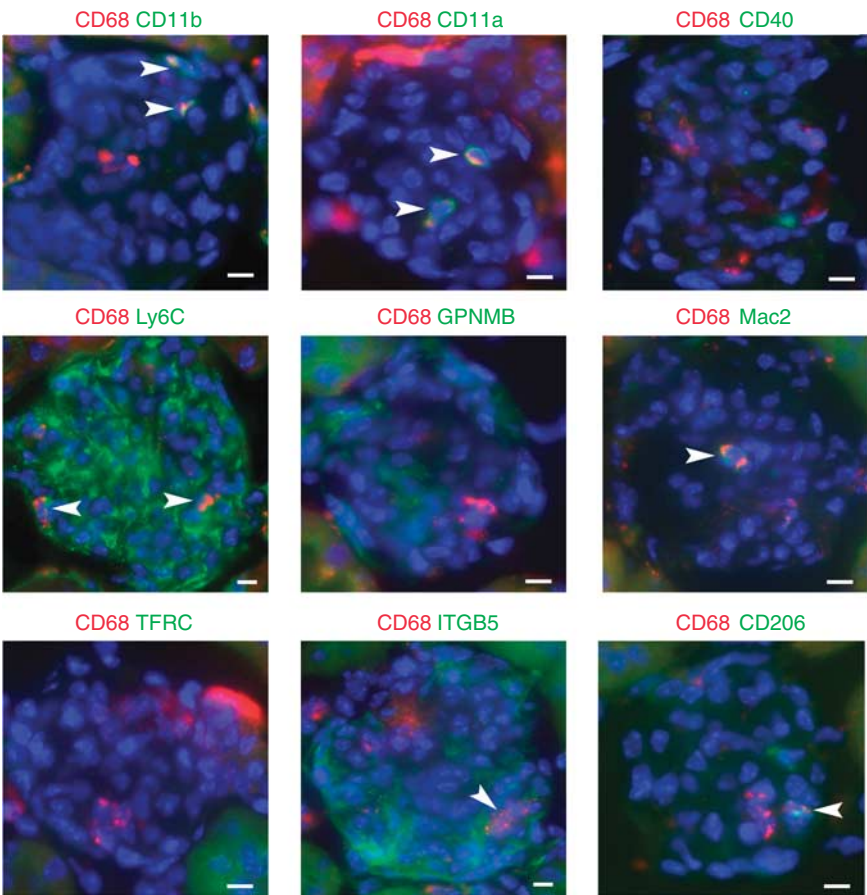


Figure 3 | Representative double immunofluorescence images of glomeruli from *Lck-TSLP* mice showing glomerular CD68-positive macrophages and expression of characteristic M1 markers CD11a and Ly6C, characteristic M2 markers Mac2 and CD206, as well as other markers expressed by macrophage subsets. Double-stained macrophages are indicated with arrowheads. Bar = 20 μm.

Table 1 | Proportion of CD68+ macrophages (Mφs) in glomerular tuft of *Lck-TSLP* mice that co-express other markers

	CD68	CD11b	CD11a	CD40	Ly6C	GPNMB	Mac-2	TFRC	ITGB5	CD206
% CD68+ Mφs	100.0	91.7	100.0	25.0	75.0	25.0	100.0	0.0	50.0	83.3
Standard error		50.0	20.0	8.3	25.0	16.7	15.0	0.0	25.0	16.7

Abbreviations: GPNMB, glycoprotein nonmetastatic melanoma B; ITGB5, integrin B5; TFRC, transferrin receptor; TSLP, thymic stromal lymphopoietin. Examples of co-expression are shown in Figure 3. Data are shown as average ± s.e. N=3 mice (10–15 glomeruli/mouse).

modest T-cell (CD3⁺) infiltration in the interstitium, but few T cells in glomeruli in any group. There was no significant difference in glomerular neutrophils assessed by Ly6G expression between the groups (neutrophils/glomerular cross-section: *CD11b-DTR-DT*: 0.059 ± 0.017; *Lck-TSLP*; *CD11b-DTR-PBS*: 0.060 ± 0.009; *Lck-TSLP*; *CD11b-DTR-DT*: 0.046 ± 0.008, not significant).

Monocyte/macrophage ablation improves kidney pathology in *Lck-TSLP*; *CD11b-DTR* mice

C57BL6 WT and *CD11b-DTR* mice treated with DT for 20 days after age 30 days did not show kidney alterations, and

were comparable with PBS-treated control mice. *Lck-TSLP* mice treated with DT for 20 days after age 30 days developed a progressive GN indistinguishable from that of *Lck-TSLP* mice treated with PBS.

Lck-TSLP; *CD11b-DTR* mice treated with DT have significantly reduced extracellular matrix expansion compared with *Lck-TSLP*; *CD11b-DTR* mice treated with PBS, as measured by silver-stained extracellular matrix (Figures 1 and 2). Type IV collagen, a major component of extracellular matrix, was also significantly reduced in the DT-treated *Lck-TSLP*; *CD11b-DTR* group; α-SMA, expressed only by activated mesangial cells, was significantly attenuated

Table 2 | Ablation of peritoneal macrophages (F4/80-positive) and blood monocytes (CD11b-positive) after administration of DT or PBS to *Lck-TSLP*; *CD11b-DTR* mice^{a,b}

Peritoneal cavity (% of total cells)	CD3	F4/80
DT	3.4 ± 1.2	4.5 ± 1.0 ^c
PBS	4.5 ± 1.5	22.7 ± 2.6
Blood (% of leukocytes)	CD3	CD11b
DT	3.5 ± 0.8	2.1 ± 0.6 ^d
PBS	3.8 ± 0.6	4.3 ± 0.7

Abbreviations: DT, diphtheria toxin; DTR, DT receptor; PBS, phosphate-buffered saline; TSLP, thymic stromal lymphopoietin.

^aPeritoneal lavage and blood are collected 24 h after intraperitoneally injected with 20 ng/g DT body weight or PBS when the age of mouse is day 30.

^bData are shown as mean ± s.e.m. (n=4 in each group).

^cP < 0.05 versus wild-type mice.

^dP < 0.001 DT-treated versus PBS-treated *Lck-TSLP*; *CD11b-DTR* mice.

by monocyte/macrophage deletion, indicating that glomerular monocyte/macrophages must activate mesangial cells (Figures 1 and 2).

Proteinuria was markedly reduced by DT treatment in *Lck-TSLP*; *CD11b-DTR* mice

Urine albumin excretion increased by fivefold in untreated *Lck-TSLP* and *Lck-TSLP*; *CD11b-DTR* mice at age 30 and 50 days. Macrophage ablation treatment markedly reduced albumin excretion in *Lck-TSLP*; *CD11b-DTR* mice (urine albumin/creatinine ratio (μg/mg); Figure 5a). Although the mice have a florid GN, renal function is preserved, as detected by serum blood urea nitrogen. Monocyte/macrophage ablation had no impact on renal function (Figure 5b).

DT treatment in *Lck-TSLP*; *CD11b-DTR* mice decreased renal TGF-β1 expression

Both *Lck-TSLP* and *Lck-TSLP*; *CD11b-DTR* mice express high transforming growth factor (TGF)-β1 transcript levels in the kidney. Monocyte/macrophage ablation decreased the TGF-β1 transcripts in *Lck-TSLP*; *CD11b-DTR* mouse kidneys (Figure 6a). Detection of TGF-β1 protein in kidney homogenates by enzyme-linked immunosorbent assay revealed a similar pattern to that of TGF-β1 transcripts: *Lck-TSLP*; *CD11b-DTR* mice with monocyte/macrophage ablation have reduced TGF-β1 protein levels compared with *Lck-TSLP*; *CD11b-DTR* mice with the normal complement of macrophages (Figure 6b).

TGF-β1 expression was restricted to the glomerular mesangium in *Lck-TSLP*; *CD11b-DTR* mice (Figure 7a) and monocyte/macrophage ablation decreased protein expression in this region (Figure 7b). Interstitial TGF-β1 was not detected in *Lck-TSLP*; *CD11b-DTR* mice.

Monocyte/macrophage ablation did not influence renal immunoglobulin or complement deposition

Immunofluorescence analysis showed that *Lck-TSLP* mice had intense deposition of IgG, IgM, and to a lesser extent of

IgA and complement C3 in glomeruli. Monocyte/macrophage ablation did not change the extent or intensity of staining of deposited immunoglobulins and complement C3 (Figure 8).

Monocyte/macrophage ablation attenuates liver, but not lung lesions, in *Lck-TSLP*; *CD11b-DTR* mice

All *Lck-TSLP* and *Lck-TSLP*; *CD11b-DTR* mice had circulating cryoglobulins, whereas WT and *CD11b-DTR* mice did not. The administration of DT did not change the cryocrit levels (a measurement of plasma cryoglobulin content) in *Lck-TSLP* or *Lck-TSLP*; *CD11b-DTR* mice (data not shown), indicating that ablation did not impact immunoglobulin production or splenic clearance. This is not surprising because splenic macrophages are not ablated in *CD11b-DTR* mice.^{15,16}

The cryoglobulinemia in *Lck-TSLP* or *Lck-TSLP*; *CD11b-DTR* mice causes systemic disease, which involves the liver and lung.^{7,8} Both *Lck-TSLP* and *Lck-TSLP*; *CD11b-DTR* mice have increased liver and lung weights. Histological evaluation of the liver showed periportal leukocyte infiltration in both *Lck-TSLP* and *Lck-TSLP*; *CD11b-DTR* mice.^{5,9} The lung lesion in *Lck-TSLP* and *Lck-TSLP*; *CD11b-DTR* mice is characterized by marked perivascular and peribronchiolar leukocyte infiltration. As assessed by Mac-2 expression, in the liver, the periportal infiltrating cells are mainly macrophages, whereas only a minority of perivascular and peribronchiolar leukocytes in the lung are macrophages (Figure 9). The hepatic periportal leukocyte infiltration was significantly reduced by administration of DT *Lck-TSLP*; *CD11b-DTR* mice (Figure 9 and Table 3). However, DT treatment had no impact on lung macrophage infiltration. Kupffer cells of normal liver and lung alveolar macrophages are also Mac-2-positive, but DT treatment did not affect these myeloid cells (Figure 9).

DISCUSSION

Monocyte/macrophages are a heterogeneous population of cells, and their effects on different diseases or even on different stages of diseases are often difficult to assess. One unresolved issue in human GN is whether the prominent monocyte/macrophage influx that characterizes cryoglobulinemic MPGN is primarily deleterious and is an effector of disease, or primarily beneficial and involved in disease stabilization and repair.

Our study showed that from the time MPGN is first detected (day 30), but when the disease is not advanced, monocyte/macrophages had mainly a detrimental role in disease progression. The specific mechanism by which monocytes/macrophages exert their deleterious effects remains unknown. The elimination of the monocyte-derived cell population had no effect on serum levels of cryoglobulinemia or on the extent of glomerular immune complex deposition. This indicates that the effect of ablation of glomerular macrophage infiltration on the kidney was due to events related to amplification of injury following immune

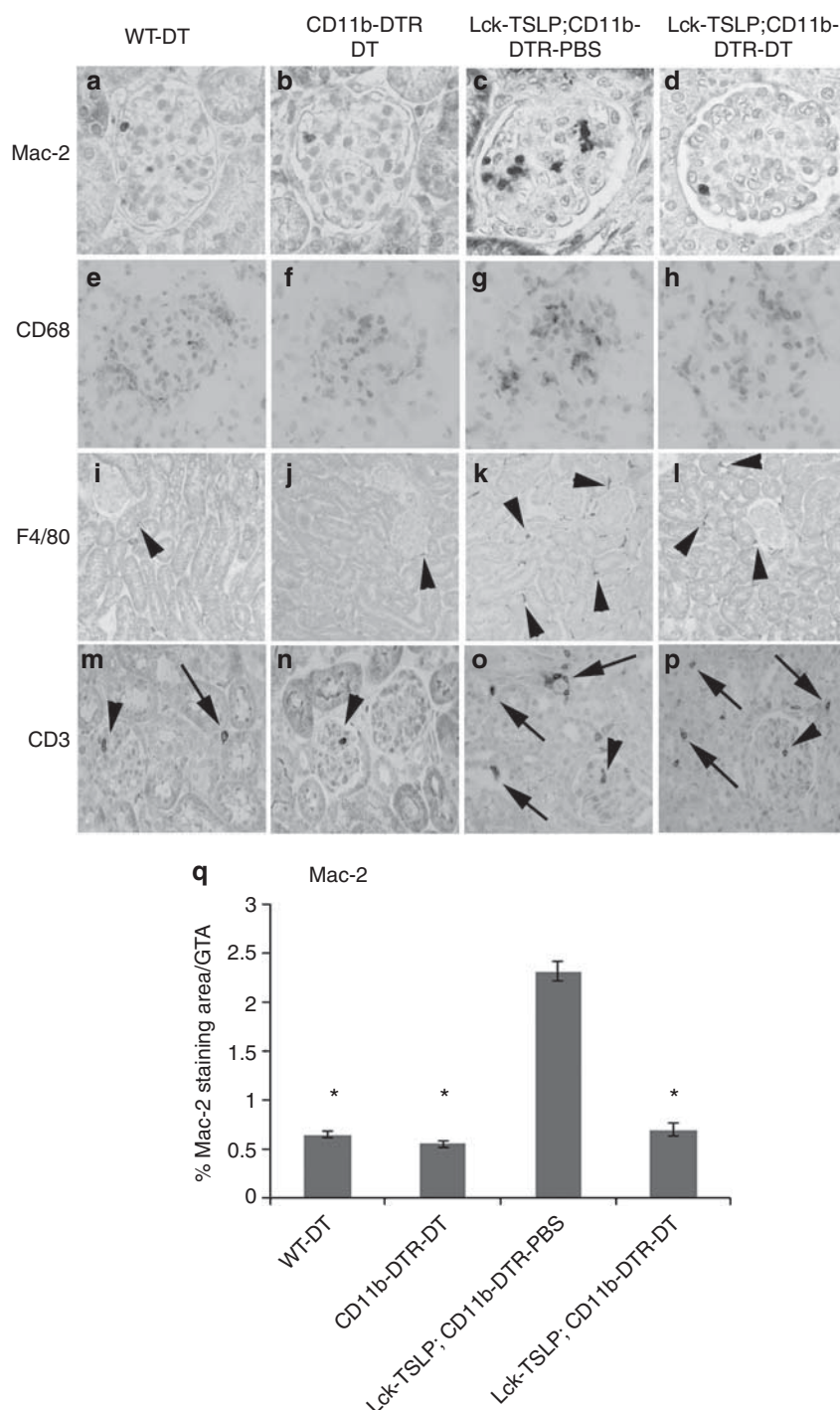


Figure 4 | Treatment of *Lck-TSLP;CD11b-DTR* mice with diphtheria toxin results in diminished numbers of glomerular macrophages, and a trend to reduced interstitial macrophages. (a) Representative photographs of expression of monocyte/macrophage markers Mac-2 (a–d), CD68 (e–h), F4/80 (i–l), and T-cell marker CD3 (m–p) in the kidneys of wild-type (WT)-diphtheria toxin (DT) (a, e, i, m), *CD11b-DTR* (b, f, j, n), *Lck-TSLP; CD11b-DTR-PBS* (c, g, k, o), and *Lck-TSLP; CD11b-DTR-DT* (d, h, l, p) mice at age 50 days, after 20 days of DT or phosphate-buffered saline (PBS) intraperitoneal administration. *Lck-TSLP; CD11b-DTR* mice show markedly increased macrophage infiltration in glomeruli (c, g) and interstitium (k; arrowheads), and increased T-cell infiltration in interstitium (o, p; arrows). *Lck-TSLP; CD11b-DTR* mice with DT treatment (d, h) have markedly reduced macrophage infiltration in glomeruli compared with *Lck-TSLP; CD11b-DTR* mice with PBS treatment (c). Original magnification: Mac-2: $\times 400$; CD68: $\times 400$; F4/80 $\times 100$; CD3: $\times 200$. (q) Morphometric analysis of glomerular macrophage infiltration is expressed as the percentage of Mac-2 staining area in the glomerular tuft area (GTA) in WT, *CD11b-DTR* mice at 50 days age with DT treatment (WT-DT and *CD11b-DTR-DT*, respectively), and *Lck-TSLP; CD11b-DTR* mice at 50 days age, after 20 days of PBS or DT treatment (*Lck-TSLP; CD11b-DTR-PBS* and *Lck-TSLP; CD11b-DTR-DT*, respectively) ($n = 5-6$ in each group). * $P < 0.001$ versus *Lck-TSLP; CD11b-DTR-PBS* group.

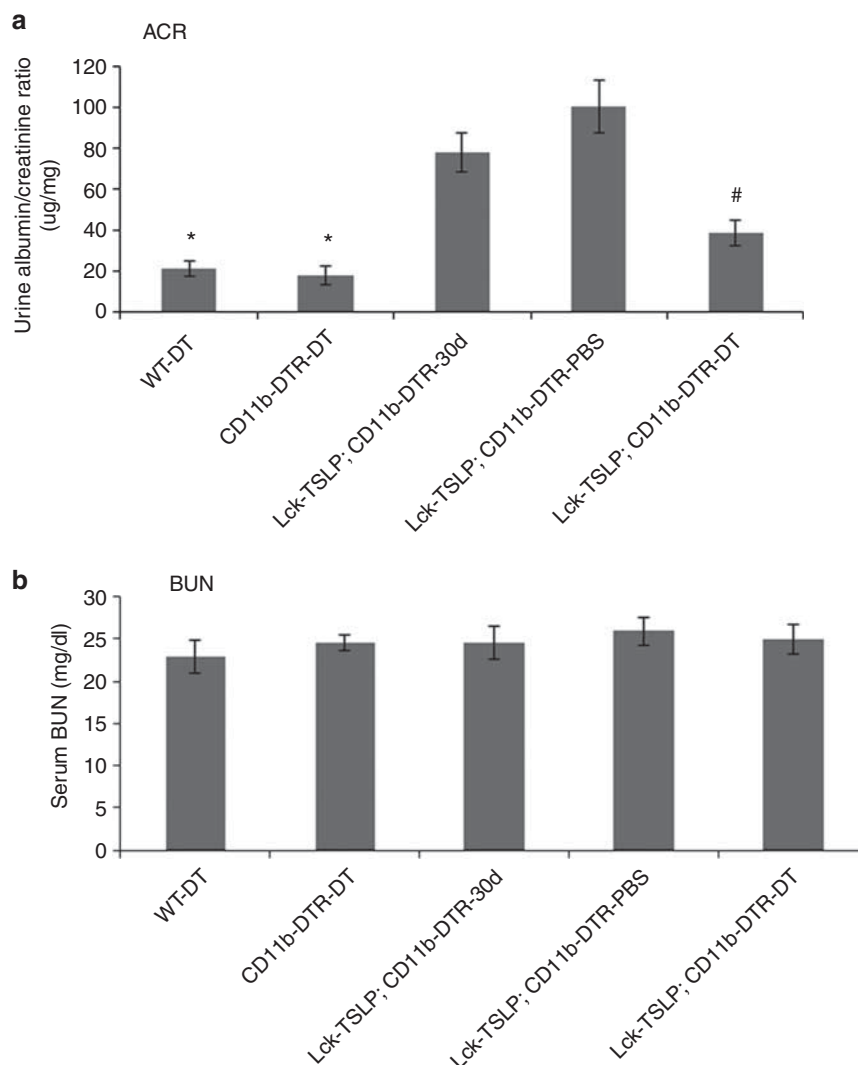


Figure 5 | Macrophage ablation reduces proteinuria in membranoproliferative glomerulonephritis. (a) Proteinuria measured as urine albumin/creatinine ratio (ACR) (expressed as µg/mg). *Lck-TSLP; CD11b-DTR* mice have markedly increased urine albumin excretion compared with wild-type (WT) and *CD11b-DTR* mice. *Lck-TSLP; CD11b-DTR* mice with diphtheria toxin (DT) treatment demonstrate significantly decreased proteinuria compared with *Lck-TSLP; CD11b-DTR* mice with phosphate-buffered saline (PBS) treatment ($n = 4-5$ each group). * $P < 0.001$, # $P < 0.01$ versus *Lck-TSLP; CD11b-DTR-PBS* group. (b) Serum blood urea nitrogen (BUN) measurements (mg/dl). There is no difference between each group ($n = 4-5$ each group).

complex deposition, and was unlikely because of a result of immune complex clearance functions performed by these cells.

We had speculated that the amplification of injury following glomerular immune complex deposition may occur by activation of infiltrating monocyte/macrophages via their Fc receptors.²⁰⁻²³ In previous studies of this model, we have shown that genetic deletion of the inhibitory FcγIIb receptor can substantially worsen disease manifestations, which may be due to loss of inhibition of B-cell functions.²⁴ Genetic deletion of the FcR γ-chain common to the activating receptors FcγIR, FcγIIIR, and FcγIVR (which results in lack of cell surface expression of these receptors) does not result in an opposite effect of amelioration of MPGN, suggesting that a key role of these activating

receptors on leukocytes is to facilitate the clearance of the deposited immune complexes, rather than activation of monocyte.²⁵ This presents a potential paradox for understanding how the loss of infiltrating monocytes/macrophages, the principal infiltrating leukocyte in cryoglobulinemic MPGN, and the cells most strongly implicated in phagocytic removal of deposited immune complexes, can still result in diminished manifestations of disease.

We have considered activities of monocytes/macrophages, other than those involved in the clearance of immune complexes, to address this paradox. For example, activation of monocyte/macrophage via Fc receptor engagement can induce production of interleukin-10, which is not only an anti-inflammatory factor but which also promotes production of growth factors for wound healing, such as TGF-β1,

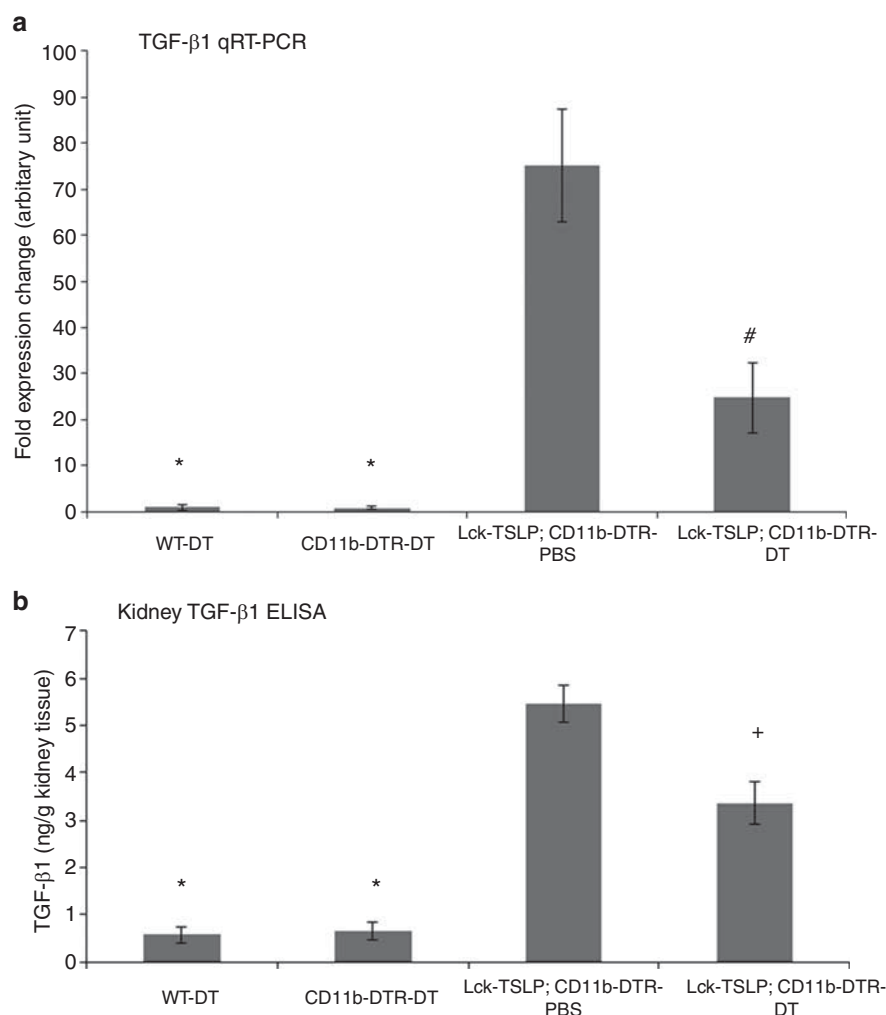


Figure 6 | Transforming growth factor-β1 levels are altered by macrophage ablation. (a) Kidney transforming growth factor (TGF)-β1 mRNA levels were reduced after diphtheria toxin (DT) treatment in *Lck-TSLP; CD11b-DTR* mice as assessed by quantitative real-time RT-PCR. The fold change of *TGF-β1* gene in *CD11b-DTR* and *Lck-TSLP; CD11b-DTR* mice was compared with wild-type (WT) mice and normalized to the endogenous *glyceraldehyde-3-phosphate dehydrogenase* gene ($n = 4$ in each group). (b) Enzyme-linked immunosorbent assay measurements of TGF-β1 protein in kidney tissue homogenates. The TGF-β1 level was normalized to the weight of the sample of the kidney cortical tissue ($n = 4$ in each group). TGF-β1 is elevated in the phosphate-buffered saline (PBS)-treated *Lck-TSLP; CD11b-DTR* mice and this elevation is reduced with DT treatment. * $P < 0.001$, # $P < 0.01$, + $P < 0.05$ versus *Lck-TSLP; CD11b-DTR*-PBS group.

which, in some tissues, may result in resolution of inflammation and healing.^{26–28} However, the continuous production of growth factors for wound healing such as TGF-β1 can be deleterious and result in progressive fibrosis or sclerosis,²⁰ and in the glomerulus it has been amply demonstrated that TGF-β1 promotes the production of glomerular matrix by mesangial cells.²⁶ Our study showed that deletion of monocytes/macrophages resulted in reduced mesangial production of TGF-β1, both at the mRNA and protein levels. Furthermore, we demonstrated that there was concurrently reduced expression of the mesangial cell activation marker α -SMA,^{29,30} and that these events correlated with markedly reduced mesangial production of type IV collagen and presumably other unmeasured components of the expanded mesangial matrix. This suggests a scenario whereby infiltrating monocyte/macrophages, communicating

with mesangial cells, initiate a cascade of events that begins with mesangial cell activation, leading to upregulated mesangial production of TGF-β1, which then acts upon these cells in an autocrine manner to drive the progressive glomerular matrix accumulation that occurs in MPGN. This scenario is then disrupted when glomerular macrophage infiltration is ablated.

An extended alternative explanation for our observations is that molecules released from injured cells of the glomerulus (endothelial, mesangial, and podocytes) serve as activating factors for recruited monocytes. These injury molecules known as danger-associated molecular patterns (DAMPs) may be responsible for the unscheduled activation of macrophages, hindering their innate function to clear immune complexes via FcγRs and complement receptors, and leading to pro-inflammatory cytokine release, which

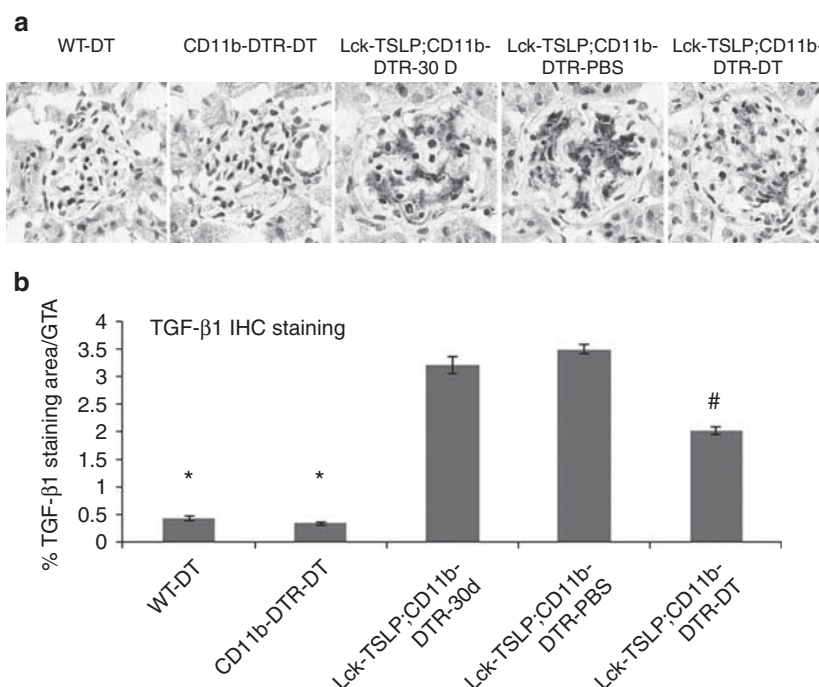


Figure 7 | Macrophage ablation results in diminished glomerular transforming growth factor- β 1. (a) Representative photomicrographs of transforming growth factor (TGF)- β 1 immunohistochemical (IHC) staining in the kidney, showing increased TGF- β 1 protein expression in glomerular mesangium of *Lck-TSLP; CD11b-DTR* mice at age 30 days and at age 50 days with phosphate-buffered saline (PBS) treatment. Diphtheria toxin (DT) treatment in *Lck-TSLP; CD11b-DTR* mice decreased glomerular TGF- β 1 protein expression. (b) Morphometric measurement of glomerular TGF- β 1 protein confirms the reduction with DT treatment. Shown is relative TGF- β 1 expression in wild-type (WT), *CD11b-DTR* mice with DT treatment at 50 days of age, *Lck-TSLP; CD11b-DTR* mice at 30 days of age, and *Lck-TSLP; CD11b-DTR* mice with PBS or DT treatment at age 50 days, after 20 days of DT or PBS intraperitoneal administration. * $P < 0.001$, # $P < 0.01$ versus *Lck-TSLP; CD11b-DTR*-PBS group ($n = 5-6$ in each group). Original magnification: $\times 400$. GTA, glomerular tuft area.

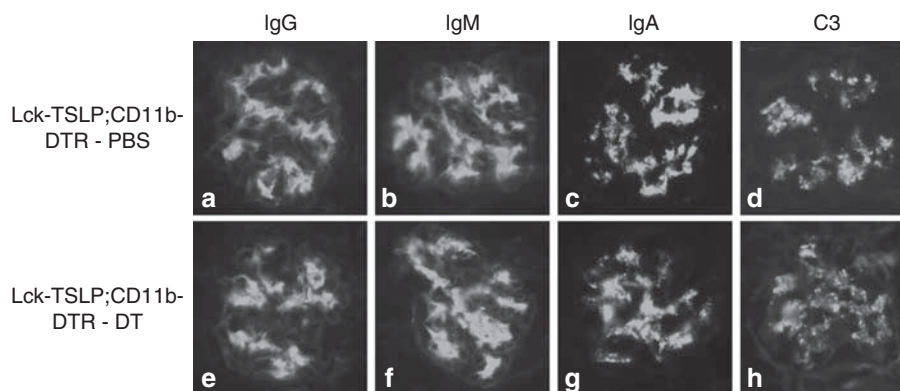


Figure 8 | Macrophage ablation did not change glomerular immunoglobulin or C3 deposition. Representative photographs of glomerular immunofluorescence in *Lck-TSLP; CD11b-DTR* mice treated with phosphate-buffered saline (PBS) (a-d) and diphtheria toxin (DT) (e-h), showing remarkable glomerular deposits of IgG (a, e) and IgM (b, f), and to a lesser extent, deposits of IgA (c, g), and complement C3 (d, h). DT treatment has no effect on glomerular immune complex deposition/accumulation. Original magnification: $\times 400$.

then drives mesangial expansion and activation as a wound healing response.³¹⁻³⁶ DAMPs can stimulate different types of activation of macrophages. Classically activated macrophages of M1 type (stimulated by bacterial cell wall molecules or interferon- γ) are widely believed to be injurious. Many DAMPs, including mitochondrial proteins and Toll-like receptor ligands, similarly activate macrophages.³¹⁻³⁶ In

contrast, macrophages stimulated by other cytokines and DAMPs including adenosine can adopt wound healing or reparative phenotypes.³⁷ We characterized the glomerular macrophages in this MPGN model and many of them expressed M1-type markers. However, they concurrently expressed markers, including Mac-2 and CD206, associated with wound-healing functions. Moreover, a minority of

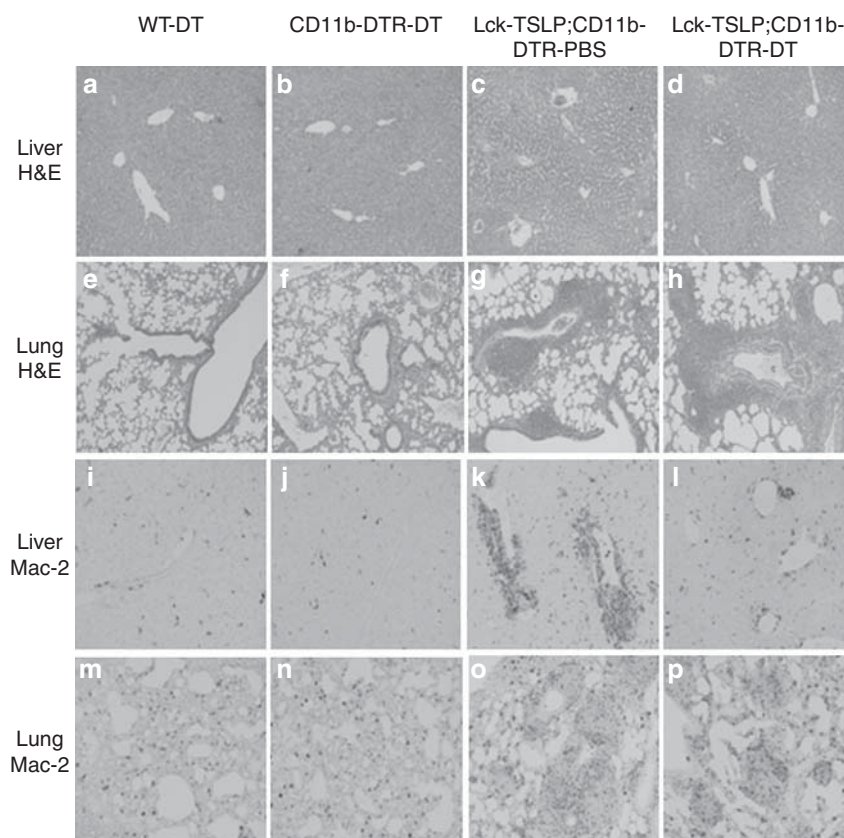


Figure 9 | Macrophage depletion has differing effects of liver and lung involvement in murine cryoglobulinemia. Representative photographs of liver (a–d) and lung (e–h) tissues stained with hematoxylin and eosin (H&E). Hepatic inflammation is significantly reduced with diphtheria toxin (DT) treatment in *Lck-TSLP; CD11b-DTR* mice. Lung inflammation is similar in distribution and in extent in both phosphate-buffered saline (PBS)- and DT-treated *Lck-TSLP; CD11b-DTR* mice. Representative photographs of liver (i–l) and lung (m–p) tissues, stained with the monocyte/macrophage marker Mac-2. In WT and *CD11b-DTR* mice, perisinusoidal Kupffer cells are Mac-2-positive. Most of the periportal infiltrating leukocytes in *Lck-TSLP; CD11b-DTR* mice are Mac-2-positive macrophages, and DT treatment resulted in significant reduction of periportal macrophage infiltration in the liver. DT treatment had no effect on the Kupffer cells. In the lung, alveolar macrophages are Mac-2-positive. Many, but not all, of the perivascular and peribronchiolar infiltrating leukocytes are monocyte/macrophages in the lung of *Lck-TSLP; CD11b-DTR* mice. Neither the alveolar macrophages nor the infiltrating monocyte/macrophages are affected by DT treatment. Original magnification: $\times 100$.

Table 3 | Semiquantitative grading of lung and liver inflammation^a

	Lck-TSLP; CD11b-DTR-30D	Lck-TSLP; CD11b-DTR-PBS	Lck-TSLP; CD11b-DTR-DT
Lung inflammation	2.0 $\pm$ 0.4	2.6 $\pm$ 0.3	2.5 $\pm$ 0.4
Liver inflammation	2.2 $\pm$ 0.5	2.4 $\pm$ 0.4	1.5 $\pm$ 0.6

Abbreviations: DT, diphtheria toxin; DTR, DT receptor; PBS, phosphate-buffered saline; TSLP, thymic stromal lymphopoietin.

^aData are shown as mean $\pm$ s.e.m. ($n=6$ in each group).

glomerular macrophages expressed glycoprotein non-metastatic melanoma B, an intracellular protein associated with reparative functions.¹⁹ This phenotypic heterogeneity may reflect transitions between one phenotype and another, but alternatively it may reflect widely differing tasks of macrophages in the glomerulus. We speculate that multiple DAMPs induced by the initial injury of cryoglobulinemic MPGN both recruit and activate these different types of macrophages. Accordingly, as *CD11b-DTR* ablation does not discriminate between activation states in the kidney,³⁸ it is

likely that the result of ablation reflects the function of most of the macrophages in the glomerular compartment. As these studies indicate that a major function of glomerular macrophages in MPGN is to activate myofibroblasts and promote matrix accumulation (M2- or wound-healing-type functions); therefore, it is not surprising that many macrophages express the M2 markers Mac-2 and CD206 and that their ablation can result in improvement in glomerular injury.

The improvement in structural glomerular injury after macrophage depletion was accompanied by functional improvement, indicated by a reduction of proteinuria. In contrast, the blood urea level was not changed in the *Lck-TSLP* and *Lck-TSLP; CD11b-DTR* mice with or without DT treatment, compared with the WT and *CD11b-DTR* mice. This may be due to the minimal tubulointerstitial injury in these mice, or due to the relative insensitivity of changes in blood urea nitrogen to glomerular disease in mice.

The effects of DT on monocyte/macrophage populations in different tissues/organs are varied. It has been shown

previously that injection of DT in *CD11b-DTR* mice can result in reduction of resident macrophages in ovaries and kidneys, but the resident lung alveolar macrophage and liver sinusoidal macrophage (Kupffer cells) are not altered.¹⁵ In the present study, both the alveolar resident macrophages and the monocyte/macrophages infiltrating lung were not sensitive to DT administration, and this intervention did not change the overall degree of lung inflammation. In the liver, the liver sinusoidal Kupffer cells were resistant to DT, but the periportal infiltration of macrophages was significantly reduced by DT administration.

In summary, we show that targeting of monocytes/macrophages, characteristically present in both human and murine cryoglobulinemic MPGN, may be an efficacious therapeutic approach for this disorder, and can result in decreased production of TGF- β 1, an established mediator of progressive glomerular injury. The influence of monocytes/macrophages on mesangial cell activation is complementary to the effects of glomerular cryoglobulin deposition, previously shown by us to be important in inducing production of serine proteases by mesangial cells.³⁹ Cryoglobulin deposition is essential for the recruitment of monocytes/macrophages in this disorder, and therefore these processes are likely synergistic in activating the mesangium at the outset of glomerular disease; however, the influence of monocytes/macrophages on mesangial cells subsequent to their recruitment is the more dominant process affecting progressive glomerular injury in the *Lck-TSLP* MPGN model. The effect on mesangial activation delineates a key activity of monocytes/macrophages in MPGN in addition to their role in disposition of deposited immune complexes, and indicates multiple roles for monocyte/macrophages in mediating this disease.¹⁵

MATERIALS AND METHODS

Animals and intervention

The experimental protocol of this study was reviewed and approved by the Animal Care Committee of the University of Washington. Mice were housed under standard conditions and received normal chow and water *ad libitum* in a specific pathogen-free facility. *Lck-TSLP* transgenic mice and *CD11b-DTR* transgenic mice, both on C57BL6 background, were crossed to develop double-transgenic mice (*Lck-TSLP*; *CD11b-DTR*). Cohorts of female C57BL6 WT, *CD11b-DTR*, *Lck-TSLP*, and *Lck-TSLP*; *CD11b-DTR* mice ($n=5-6$ in each group), were studied.

Diphtheria toxin (1.0 mg) (List Biological Laboratories, Campbell, CA) was reconstituted with 1.0 ml sterile distilled water and then diluted to 2 ng/ml in sterile PBS. Mice at age 30 days, when kidney disease is early in its evolution in female *Lck-TSLP* mice as previously documented, were injected intraperitoneally with 20 ng/g DT body weight or similar volume of vehicle (PBS), every other alternate day for 20 days.

Tissue collection and histological staining

The genotyped groups of mice ($n=5-6$ in each group) were analyzed at 30 days and 50 days after birth following 20 days of DT or PBS treatment. Blood was collected under an anesthetic before collecting kidney, liver, and lung. Portions of each organ were fixed

in 10% neutral-buffered formalin or methyl Carnoy's solution. These tissues were processed, embedded in paraffin, and histological sections were prepared using standard procedures. Tissue sections were stained with hematoxylin and eosin, periodic acid-Schiff, and silver methenamine reagents (silver staining). Portions of cortical kidney were snap-frozen and stored in -80°C for subsequent isolation of RNA and protein. Portions of kidney in Tissue-Tek OCT compound (Sakura Finetek USA, Torrance, CA) were snap-frozen and stored at -80°C , and sectioned at 5 μm , fixed in cold acetone or methanol for 10 min, and then used for immunofluorescence analysis. Both frozen and paraffin-embedded tissues were used in immunohistochemistry studies. In some experiments, organs were fixed in PLP solution before cryosectioning as previously described.^{40,41}

Immunostaining and immunofluorescence

The general immunohistochemistry protocols used have been described previously.^{5,24,40,41} Type IV collagen was detected in paraffin-embedded tissue by a goat polyclonal anti-human type IV collagen antibody that cross-reacts with mouse type IV collagen (Southern Biotechnology, Birmingham, AL).^{7,8} Glomerular mesangial cell activation was evaluated in paraffin-embedded tissue by α -SMA expression.^{7,30} Tissues were first blocked by Rodent Block M (Biocare Medical, Concord, CA) and then incubated with a mouse monoclonal anti-human α -SMA antibody (Dako, Carpinteria, CA). After washing, MM-HRP Polymer (Biocare Medical) was added and then detected with DAB substrate. Monocytes/macrophages were detected by a rat anti-mouse Mac-2 antibody (Cedarlane, Ontario, Canada) in paraffin-embedded kidney, liver, and lung tissues.^{7,8} Monocytes/macrophages in the kidney were also detected by a rat anti-mouse CD68 antibody (AbD Serotec, Raleigh, NC) using frozen kidney tissue and F4/80 (AbD Serotec) in paraffin tissue sections. T cells and neutrophils were identified using rat anti-mouse CD3 antibody (AbD Serotec) and rat-anti-Ly6G antibody (eBioscience, San Diego, CA), respectively, in paraffin-embedded tissue. Kidney TGF- β 1 expression was evaluated by staining with a rabbit anti-mouse TGF- β 1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) on frozen kidney tissue.^{7,8,42} To detect immunoglobulins, acetone-fixed cryosections were air-dried and washed in PBS, then incubated with fluorescein-conjugated antibodies against mouse IgG, IgM, IgA, and complement C3 (Cappel Pharmaceuticals, Aurora, OH) as previously described,^{7,8} and then mounted in Vectashield (Vector, Burlingame, CA). In a blinded manner, the glomerular fluorescence intensity was described semiquantitatively (0, negative; 1, weak; 2, moderate; 3, strong). For every sample, 15 glomeruli were evaluated and the mean score was calculated.

In PLP-fixed cryosections, macrophages and macrophage subpopulations were detected using methods previously reported^{40,41} by antibodies against the following epitopes: CD68 and CD68-FITC (1:200 AbD Serotec), CD11b, CD11b-FITC (1:400 eBioscience), CD11a, Ly6C, CD206-FITC, CD40 (1:200 BD Pharmingen, San Diego, CA), Mac-2-FITC (1:200 Cedarlane), transferrin receptor, integrin β 5 (1:200 Invitrogen, Carlsbad, CA), and glycoprotein non-metastatic melanoma B (1:1000).¹⁹ Where appropriate, affinity-purified anti-rat-Cy3 or anti-rabbit-Cy3 antibodies (1:800 Jackson ImmunoResearch, West Grove, PA) were applied. After washing and post fixation (1% paraformaldehyde), sections were mounted with Vectashield containing 4',6'-diamidino-2-phenylindole. For double staining, unlabeled primary antibody followed by Cy3-conjugated secondary antibody was followed by a second FITC-conjugated antibody recognizing CD68 or CD11b.

Analysis of renal function

Before collection of mouse organs, spot urine was collected. Urine albumin level was measured by enzyme-linked immunosorbent assay, (Albuwell, Exocell, Philadelphia, PA) and urine creatinine level was measured using the Creatinine Companion kit (Exocell) according to protocols of the manufacturer. Proteinuria level was evaluated by urine albumin/creatinine ratio. Serum blood urea nitrogen level was measured with the liquid urea nitrogen reagent set (Pointe Scientific, Canton, MI).

Measurements of serum cryoglobulins

Blood was allowed to clot at 37 °C and serum was collected by centrifugation (3000 r.p.m., 10 min). Serum was stored at 4 °C for a minimum of 72 h and the formation of cryoprecipitate was identified and serum cryoglobulin concentration (cryocrit) was measured as previously described.^{5,7}

Quantitative real-time PCR and enzyme-linked immunosorbent assay measurements of TGF- β 1 in kidney tissue

Portions of frozen cortical kidney tissue were weighed, homogenized, and total kidney RNA and protein was extracted using the PARIS kit (protein and RNA isolation system) (Ambion, Austin, TX). The isolated mRNA was converted to cDNA using Retroscript RT-PCR kit (Ambion). Real-time PCR was performed using Taqman gene expression assay kits for the primers and probes of mouse TGF- β 1 gene and glyceraldehyde-3-phosphate dehydrogenase gene (Applied Biosystems, Foster City, CA). The fold change of the expression level of TGF- β 1 transcript in *Lck-TSLP* mice compared with WT mice was normalized to the endogenous housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. The isolated protein homogenates were used for TGF- β 1 protein measurement using TGF- β 1 enzyme-linked immunosorbent assay (eBioscience). Total TGF- β 1 protein was normalized to the weight of the kidney tissue from which the protein was extracted.

Peritoneal macrophages and blood monocyte ablation test

Twenty-four hours after intraperitoneal injection with 20 ng/g DT body weight or similar volume of vehicle (PBS), when the mouse was 30 days of age, peritoneal cells were collected by peritoneal lavage with 3 ml of PBS. Blood was collected retro-orbitally and was treated with red blood cell lysis buffer (Invitrogen). Cells were blocked in FcR Block (BD Pharmingen). Anti-mouse CD11b-FITC and anti-F4/80-FITC (AbD Serotec) were used as markers for monocytes in blood and macrophages in peritoneal cavity, respectively, and anti-mouse CD3-PE (BD Pharmingen) was used as marker for T cells, were added in 50 μ l total volume and incubated at 4 °C for 30 min. Cells were washed in FACS buffer three times followed by flow cytometry.

Glomerular and interstitial morphometry

Fifteen random glomerular cross-sections were photographed using an Olympus DP11 digital camera (Olympus, Melville, NY). Five non-overlapping, low-power cortical fields were photographed to quantify interstitial macrophages. Photography and subsequent morphological analyses were performed by an examiner blinded to the origin of the samples. The photographs were imported and analyzed using the ImagePro Plus software (Media Cybernetics, Silver Spring, MD). Glomerular tuft area and the proportion of the glomerular area occupied by black silver methenamine-stained matrix or stained by antibodies to type IV collagen, TGF- β 1, or the

proportion occupied by cells expressing α -SMA, or Mac-2 were quantified as previously described.^{6-8,24} Similarly, cortical area and the area occupied by cells expressing F4/80 were quantified. Ly6G-positive neutrophils were counted in a minimum of 75 glomerular cross-sections.

Analysis of extrarenal involvement

Liver and lung tissue sections stained with hematoxylin and eosin were assessed for evidence of systemic injury. As previously described, the liver and lung inflammation was scored semiquantitatively on a scale of 0–3: 0, no inflammation; 1, mild; 2, moderate; 3, severe with regard to the extent and density of leukocytic infiltration.^{7,8}

Statistical analysis

All data are expressed as mean $\pm$ s.e.m. Statistical analysis of the data for multiple groups was performed using analysis of variance with Tukey–Kramer multiple comparisons test using the InStat program (Version 3.0, Intuitive Software for science, San Diego, CA). $P < 0.05$ was considered significant.

DISCLOSURE

All the authors declared no competing interests.

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